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Separated components of root exudate and cytosol stimulate different morphologically identifiable types of branching responses by arbuscular mycorrhizal fungi

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ABSTRACT

Two morphologically distinct hyphal branching responses by the AM fungus, *Glomus intraradices*, were stimulated by separated components of carrot root exudate. Complex branching up to the sixth order was induced by compounds most soluble in 35 % methanol, whereas the formation of more lateral branches (second order) was stimulated by compounds most soluble in 70 % methanol. This same 70 % alcohol soluble fraction also stimulated a completely different type of branching pattern in another fungus, *Gigaspora gigantea*. This pattern consisted of a very periodic distribution of dense clusters of hyphal branches that had a very high degree of complexity. In contrast to exudate components, separated cytosolic components of carrot roots did not stimulate any of the observed hyphal branching patterns. Alcohol-soluble fractions actually inhibited hyphal tip growth of *G. gigantea* and induced the formation of ''recovery'' branches that were identical to those induced by an inhibitor found in the exudate of Chard (*Beta vulgaris* ssp. cicla), a non-host plant.

Introduction

The first necessary interaction between an AM fungus and a host root is an increase in hyphal growth/branching by the fungus in response to chemical components found in the host root exudates (Elias & Safir 1987; Garriock *et al.* 1989; Giovannetti *et al.* 1993). A previous experimental system demonstrated that hyphal branching and growth were stimulated, although the fungus was physically separated from the host root by a membrane filter (Giovannetti *et al.* 1993). This result clearly indicated that a branching stimulator or factor (BF) could readily diffuse from the root surface and was confirmed by an *in vitro* system in which partially purified exudate components induced branching of germinated spores of *Glomus rosea*, *G. margarita* (Buee *et al.* 2000), *G. intraradices*, and *Gigaspora gigantea* (Nagahashi & Douds 2000).

A recent report has identified strigolactone (5-deoxystrigol) as a BF that stimulates hyphal branching of *G. margarita* (Akiyama *et al.* 2005). Strigolactone is a type of sesquiterpene, which was found in the exudates of *Lotus japonicus* and some of these compounds were previously identified as seed-germination stimulants for the parasitic weeds *Striga* and *Orobanche* (Bouwmeester *et al.* 2003). Strigolactone is unlikely to be the only BF that stimulates AM fungal growth because a previous report showed that multiple active compounds from a root exudate could be separated by t.l.c. (Nagahashi & Douds Jr

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2000). We report here that separated chemical components from a carrot root exudate can induce morphologically distinct hyphal branching patterns when applied to germinated spores of AM fungi. In addition, a morphologically distinct hyphal tip inhibition/recovery pattern is induced by components of both carrot cytosol and Chard root (non-host) exudate.

Materials and methods

Spore production and germination

Azygospores of *Gigaspora gigantea* were produced in pot cultures in a greenhouse using *Paspalum notatum* as a host. The pot culture soil was air-dried and stored at 4 °C for four months to relieve dormancy of the spores. Spores were isolated by wet sieving and centrifugation (Gerdemann & Nicolson 1963; Jenkins 1964), sterilized as described by Bécard & Fortin (1988), and stored at 4 °C until use. The spores were then transferred to Petri dishes containing M medium (Bécard & Fortin 1988) solidified with 0.4 % gellan and placed in an incubator at 32 °C in a 2 % CO_2 atmosphere. After germination, individual spores were transferred in a plug of medium to a Petri dish of fresh M medium and allowed to grow for 3–6 d before testing for hyphal branching stimulators. Spores from *Glomus intraradices* (DAOM 181602) were obtained from a split culture plate technique described previously (St-Arnaud *et al.* 1996).

Intact plants, root cultures and exudates

Transformed (Ri T-DNA) carrot roots (*Daucus carota*) grown in liquid culture were used to produce the exudates as described (Nagahashi & Douds 2000). Roots were grown for two weeks in liquid M medium at 24 °C in a shaking water-bath. The medium was then harvested and either replaced with normal liquid medium with 35 μM phosphorus (+Pi) or M medium without Pi (−Pi) and the roots were allowed to grow an additional week. At this time the exudates were harvested and concentrated in a C18 Sepak cartridge (0.5 g), eluted first with 3 ml of 35 % acetonitrile (v/v) to remove the yellow brownish colour (this was more effective than using 35 % methanol), followed by 3 ml 70 % acetonitrile and finally 3 ml 100 % acetonitrile. These fractions contained the compounds of interest and were dried under a stream of nitrogen and stored frozen until used. The frozen dried samples were dissolved in 1 ml of the appropriate concentration of methanol, rather than acetonitrile, which could not be used in the bioassays. The original percent acetonitrile fractions were replaced by the same percent in alcohol. Therefore, the fractions will be identified as the 35 % alcohol soluble fraction (ASF), 70 % ASF, and 100 % ASF.

Chard (*Beta vulgaris* ssp. *cicla*), a non-host plant, was grown in a greenhouse. Twenty-five rainbow chard seeds were planted in 4″ pots filled with vermiculite. The pots were watered daily and given full-strength Hoagland's solution once a week (Hoagland & Arnon 1938). Plants were allowed to grow for three weeks, removed from the pots, and roots were rinsed in water to remove the vermiculite. The roots of the intact plants were placed in a beaker containing 80 ml water and allowed to sit for 3 h at room temperature to collect the exudate. The exudate was concentrated on a C18 cartridge and fractionated exactly like the *in vitro* carrot root exudate described above.

Root cytosolic components

Cultured carrot roots were washed three times with cold, deionized distilled water to remove soluble compounds (remaining exudate) from the apoplast or cell wall freespace. Roots (8 g fresh weight) were then ground with a mortar and pestle in 0.1 M Tris–HCl, Trizma Base at pH 7.5 with 5 mM dithiothreitol at 4 °C. The homogenization ratio of grinding medium (ml) to fresh weight of roots (g) was 10:1. The homogenate was strained through cheesecloth and centrifuged at 80000 ***g*** for 45 min at 4 °C to pellet the organelles and microsomes. The resulting supernatant contained the soluble cytosolic compounds. For plant tissue, the term cytosol is loosely used as it also contains soluble compounds from the vacuole. The cytosolic fraction was diluted to 250 ml with distilled water, passed through a C18 cartridge, and fractionated the same way as the exudate. The dried fractions were dissolved in 1 ml of the appropriate alcohol concentration so the activity in the exudate and cytosol were assayed at the same level relative to the fresh weight of roots.

Bioassays for BFs and inhibitors

When *Gigaspora gigantea* was used as the test organism, the bioassay was performed as described earlier (Nagahashi & Douds 1999) except that 8 μl of sample was injected per well. The assay was usually terminated 3 d after the injections by pouring a Trypan blue stain (0.47 g trypan blue, 250 ml lactic acid, 417 ml glycerol, 250 ml H_2O) over the gel. For *G. intraradices*, nine to 12 spores were inserted into round Petri plates (100 × 15 cm) containing M medium which was solidified with gellan. The spores usually germinated in 4–6 d. When some of the spores germinated, the new hyphal growth from the subtending hypha curled and stayed near the spore and no major hyphae could be readily identified. These spores were not treated as the analysis was impossible to do. Germinated spores with a dominant germ tube hypha growing away from the spore were chosen for treatment. Various ASFs were injected into wells placed near this major hypha and growth was terminated after 10 d as described above. When the hyphal branching patterns of both species were compared, the same sample and same volume (8 μl per well) were used. Quantification was done by counting the number of branches at ×20 with a stereomicroscope (Zeiss STEMI SR) after the hyphae were stained. Overall growth measurements were made by using the grid-line intersect method (Newman 1966).

A Nikon DXM1200 F microscope was used to count the number of branches in the area between the tip and the origin of the main germ tube coming out of the subtending hypha of the spore. Hyphal branches arising from this major hypha were defined as secondary branches (2°), those arising from the 2° were defined as 3°, and so forth. Spore germination and hyphal growth after various treaments were performed in a carbon dioxide (2.5 %) incubator to maximize growth (Bécard & Piché 1989).

Results

The partially separated carrot root exudate components induced two morphologically distinct hyphal branching patterns in *Glomus intraradices*. The 35 % soluble fraction stimulated the highest order of branching as shown in Fig 1. The level of branching often reached the 6° and occasionally the 7°. In contrast, the fraction soluble in 70 % alcohol induced the highest numbers of lateral or 2° branches (Fig 1). Micrographs of the morphologically different branching patterns between the control, 35 % ASF, and 70 % ASF are clearly shown in Fig 2. A 1:10 dilution of the 35 % ASF decreased the number of branches that grew off a major hypha, but the branches that were induced had up to the 4° to 5° order of branching (data not shown). When the 70 % ASF was diluted 1:10, fewer lateral branches were observed and the morphology was identical to the control (Fig 2A) with little or no branching at the 3° level (data not shown).

When the same 70 % fraction used on *G. intraradices* was applied to germinated spores of *G. gigantea*, a completely different branching pattern was induced. The pattern consisted of repetitive clusters of branches and to represent their overall periodical distribution, the branching pattern had to be traced directly on the culture plate (Fig 3A).

Unlike the hyphal growth of *G. intraradices*, which was small enough to take a micrograph of a good portion of a major hypha, *G. gigantea* has a much greater overall growth pattern, so only a small portion of the branching pattern of the primary germ tube could be shown in a single micrograph (Fig 3B). Each cluster had a tremendous amount of branching, which resembled a bush, and the highest order of branching (Fig 3C) was not determined.

In contrast, the isolated components (35 %, 70 %, and 100 % ASF) of carrot root cytosol (cytoplasm with the organelles removed) did not stimulate any of the aforementioned branching patterns of *G. gigantea*. The 100 % ASF had no effect on hyphal branching or growth but the 35 % and 70 % ASFs (data not shown) inhibited hyphal growth. The concentrated 35 % ASF inhibited all treated hyphal tips and even the 'recovery' branches that formed did not continue to grow (Fig 4A). However, treatment with a diluted sample (1:5 dilution) allowed the 'recovery' branches behind an inhibited tip (Fig 4B) to continue to grow. This inhibition of hyphal tip growth with the generation of 'recovery' branches occurred on both the primary germ tube and major secondary hyphae (Fig 4C), but it was most apparent on the 1° germ tube as the recovered hyphal branch (s) became negatively geotropic. The average number of recovery branches on a primary germ tube was three (15 observations), whereas the control showed no recovery branches. At dilutions

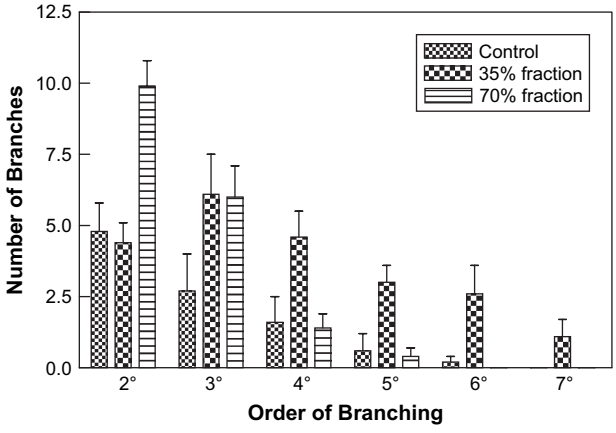

Fig 1 – Stimulation of hyphal branching of *Glomus intraradices* induced by separated components of carrot root exudate with different solubilities in methanol. The major hypha (1°) had branches (2°), which had branches (3°), etc. The number of branches between the tip of the major hypha and the subtending hypha of the spore were counted for each treatment. Means of ten observations ± SEM. This graph is one of three separate experiments, which all showed similar results.

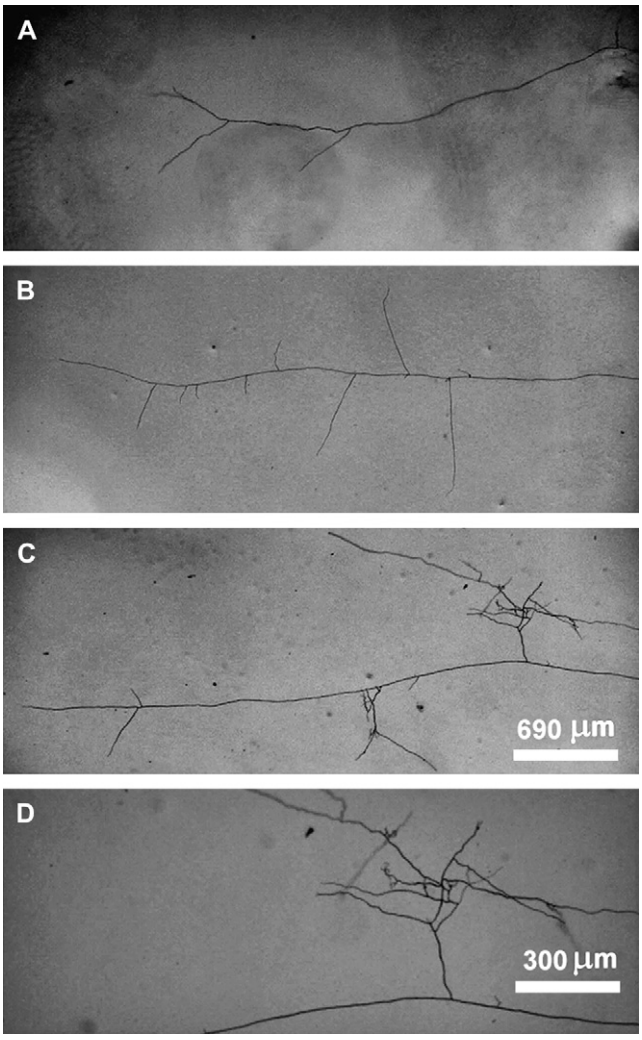

Fig 2 – Micrographs of the hyphal branching morphology of *Glomus intraradices* induced by alcohol soluble fractions from carrot root exudate. Each micrograph shows approximately two thirds of the distance between the major hyphal tip and the subtending hypha of the spore. (A) Major hypha treated with 70 % methanol as a control (B). Major hypha treated with a 70 % ASF. (C) Major hypha treated with a 35 % ASF. (D) A higher magnification of the hyphal branching shown in (C). Note the greater degree of secondary branches in (B) compared with the more complex branching in (C). Figs (A), (B), and (C) are all the same magnification.

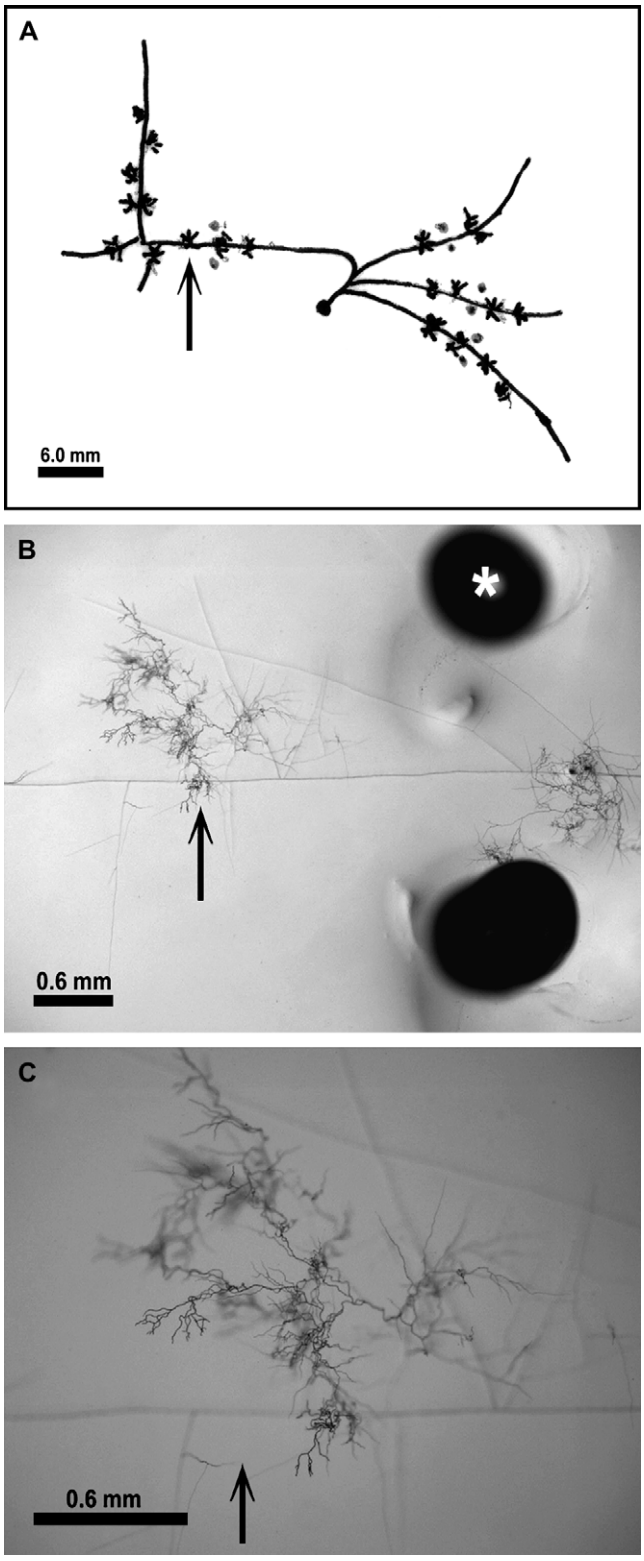

Fig 3 – A tracing and micrographs of the branching pattern of *Gigaspora gigantea* induced by the 70 % ASF of carrot root exudate. (A) To show the overall periodical distribution of hyphal clusters, a tracing was made directly on the culture dish. (B) Actual micrograph of the hyphal cluster as indicated by the arrow in the tracing. The dark circle marked by the asterisk is one of the holes placed in the gel where the exudate components were injected. (C) Micrograph of the hyphal cluster shown in (B) taken at a higher magnification. The highest degree or order of branches was not determined for this very complex 'bush'-like branching pattern.

of 1:25 or 1:50, no recovery branches or hyphal tip inhibition was observed and the average growth and number of branches were the same as the controls.

The same morphological response was induced by the 35 % ASF of the exudate collected from chard roots, a non-host plant. The concentrated form inhibited the tips of the primary germ tube, as well as the tips of all secondary hyphae of *G. gigantea* and no recovery of growth was observed (micrograph not shown). Dilution of the non-host exudate (1:10) again inhibited hyphal tip growth but the recovery branches generated behind the inhibited tip (Fig 4D) continued to grow. The hyphal branch pattern induced by carrot cytosol components (Fig 4B–C) and chard exudate components (Fig 4D) were virtually identical.

When germinated spores of *G. intraradices* were treated with the 35 % ASF from the carrot cytosol, no apparent recovery branches were observed. However, the treatment inhibited both the overall length and number of branches by over 50 % (Table 1). A very similar inhibition of growth and branches was observed when germinated spores were treated with the 35 % ASF from chard exudate (data not shown).

Discussion

The first result reported here clearly indicated that partially purified BFs in carrot root exudates can stimulate morphologically distinct types of hyphal branching patterns (Fig 2) within a species (*Glomus intraradices*). Furthermore, another species (*Gigaspora gigantea*) responded to the same fraction (70 % ASF) with a different and distinct morphological branching pattern (Fig 3). These results confirm an earlier report (Nagahashi & Douds 2000) that demonstrated the presence of multiple BFs in carrot root exudates. The data presented here also indicate that either any given AM fungus species may have a unique reaction to the same BF or different AM species react more favourably toward one BF versus another. Regardless of the type of interaction, the various types of branching patterns all provide for a greater chance of contact between the AM fungus and host root surface. The chemical compounds or chemical categories these BFs represent in carrot root exudates are still unknown. Although one hyphal BF from the exudate of *Lotus japonicus* (Akiyama *et al.* 2005) and *Sorghum* (Besserer *et al.* 2006) has been identified as 5-deoxy-strigol, this compound has not been reported to be present in carrot root exudate. Possible derivatives of strigolactone may be present as determined indirectly with a seed germination bioassay (Yoneyama *et al.* 2006).

The second major observation reported here was the presence of a hyphal growth inhibitor instead of a stimulator within the cytosol of carrot roots. It is unlikely that the presence of this inhibitor or inhibitors was masking the activity of BFs because after the 35 % ASF and 70 % ASF (data not shown) were diluted, no hyphal branching beyond that of the control was observed. These component(s) of the cytosol may be compounds of the host-defence system, which are antimicrobial in nature. It is also possible that the BFs are

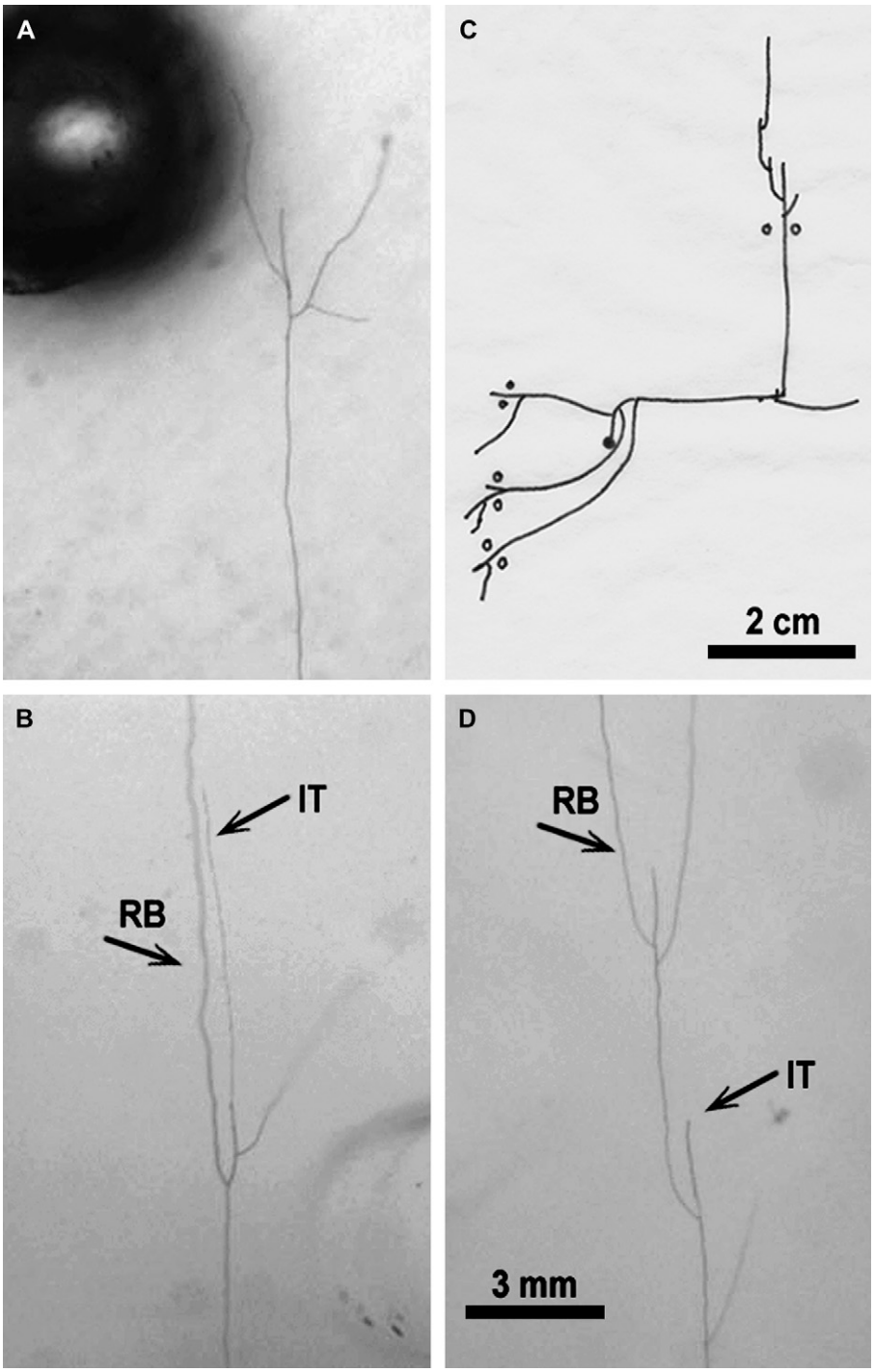

Fig 4 – Micrographs of hyphal tip inhibition of *Gigaspora gigantea* by the 35 % ASF of carrot root cytosol (A–C) and the 35 % ASF of a non-host chard root exudate (D). (A) The hyphal tip of the primary germ tube was inhibited (IT) and although recovery branches formed, the concentration of inhibitor did not allow the AM fungus to recover. (B) A 1:5 dilution of the 35 % ASF showed the successful continuation of the growth of a recovery branch (RB) after the initial inhibition of the germ tube tip. (C) The tracing shows the inhibition and recovery branches of both the primary and major secondary hyphae after treatment with the diluted cytosolic fraction. (D) A 1:10 dilution of the concentrated sample from the exudate of chard roots showed the formation of recovery branches. The tip of a recovered hypha was often inhibited and another recovery branch was formed, etc (see arrows). Eventually the inhibitor was diluted enough that a recovery branch finally resumed normal growth. Figs (A), (B), and (D) are all at the same magnification.

Table 1 – The inhibition of growth of germinated spores of *Glomus intraradices* after treatment with the 35 % methanol soluble fraction isolated from carrot root cytosol

Treatment	Total length (mm)	Total number of branches
Control	5.8 ± 0.9	18.9 ± 3.3
Carrot cytosol	2.7 ± 0.5	9.3 ± 2.2

The data are from ten observations (± SEM).

present in the cytosol, but have an attached R-group that renders these compounds inactive or even inhibitory. Whatever the case, the inhibited tip/recovery growth response (Fig 4A–C) was the same as that generated by a component of a non-host (chard) exudate (Fig 4D). Both systems inhibited tip growth of the primary germ tube of *G. gigantea* and induced formation of a recovery branch behind the tip. If the new tip was inhibited, another recovery branch was formed and this unique type of response resembled a 'candelabra' (Fig 4). The same recovery response was found when a germinated spore was placed near the root system of an intact chard plant *in vitro* (data not shown) and Ri T-DNA transformed sugar beet roots (Nagahashi & Douds 2000). In the presence of non-host roots or exudate, the inhibition was overcome and normal hyphal growth resumed when one of the recovery branches grew further away from the intact root or when the presence of an exudate inhibitor was diluted as a result of diffusion. The formation of recovery branches could be interpreted as branching stimulation, however, the pattern is more of an avoidance response (Fig 4) as also suggested in the data on *Glomus mosseae* in the presence of *Brassica* roots (Glenn *et al.* 1988).

Finally, the lack of BFs in the cytosol of the host roots poses an interesting problem. This observation means that compounds that stimulate hyphal branching are selectively exuded or activated. It is unlikely that these compounds are constitutive components of the plant cell wall, which are released during the interaction with the fungus, as the BFs are present in the exudate even when the roots have never been exposed to an AM fungus. If the BFs are in an inactive form within the cell, they would have to be activated either during the secretory process or after secretion by specific host cell wall-associated enzymes or even enzymes in the soil. The presence of a side group may not only inactivate a BF but may also mark the compound for an extracellular destination. Hydrolysis of the R group from a BF in secretory vesicles would activate the compound as it is secreted via the endomembrane system. What actually makes a BF unique to the exudate is an intriguing question and needs to be addressed in terms of a compartmentation of secondary metabolism and possible compartmentation/modification of secondary metabolites.

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